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Extracellular Matrix Assembly in Diatoms (Bacillariophyceae)[1]

II. 2,6-Dichlorobenzonitrile Inhibition of Motility and Stalk Production in the Marine Diatom *Achnanthes longipes*

Yan Wang, Jingjie Lu, Jean-Claude Mollet, Michael R. Gretz*, and Kyle D. Hoagland

Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931–1295 (Y.W., J.L., J.-C.M., M.R.G.); and Department of Forestry, Fisheries, and Wildlife, University of Nebraska, Lincoln, Nebraska 68583 (K.D.H.)

The cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) and the DCB analogs 2-chloro-6-fluorobenzonitrile, 3-amino-2,6-dichlorobenzonitrile, and 5-dimethylamino-naphthalene-1-sulfonyl-(3-cyano-2, 4-dichloro)aniline (DCBF) inhibited extracellular adhesive production in the marine diatom *Achnanthes longipes*, resulting in a loss of motility and a lack of permanent adhesion. The effect was fully reversible upon removal of the inhibitor, and cell growth was not affected at concentrations of inhibitors adequate to effectively interrupt the adhesion sequence. Video microscopy revealed that the adhesion sequence was mediated by the export and assembly of polymers, and consisted of initial attachment followed by cell motility and eventual production of permanent adhesive structures in the form of stalks that elevated the diatom above the substratum. *A. longipes* adhesive polymers are primarily composed of noncellulosic polysaccharides (B.A. Wustman, M.R. Gretz, and K.D. Hoagland [1997] Plant Physiol 113: 1059–1069). These results, together with the discovery of DCB inhibition of extracellular matrix assembly in noncellulosic red algal unicells (S.M. Arad, O. Dubinsky, and B. Simon [1994] Phycologia 33: 158–162), indicate that DCB inhibits synthesis of noncellulosic extracellular polysaccharides. A fluorescent probe, DCBF, was synthesized and shown to inhibit adhesive polymer production in the same manner as DCB. DCBF specifically labeled an 18-kD polypeptide isolated from a membrane fraction. Inhibition of adhesion by DCB and its analogs provides evidence of a direct relationship between polysaccharide synthesis and motility and permanent adhesion.

The herbicide DCB is considered a specific inhibitor of cellulose synthesis in higher plants and algae (Delmer and Amor, 1995). Disruption of plant growth in the presence of DCB has been demonstrated in cotton (Montezinos and Delmer, 1980; Delmer et al., 1987), tobacco (Meyer and Herth, 1978), tomato (Shedletzky et al., 1990), barley (Shedletzky et al., 1992), the charophytes *Chara* and *Nitella* (Foissner, 1992), and the green alga *Vaucheria* (Mizuta and Brown, 1992). At micromolar concentrations effective at inhibiting cellulose synthesis, DCB appears to have little or no short-term effect on the synthesis of noncellulosic polysaccharides, nuclear division or DNA synthesis, protein synthesis, respiration, or the in vivo labeling patterns of UDP-Glc, phospholipids, or nucleoside mono-, di-, or triphosphates (Delmer, 1987, and refs. therein).

The exact mode of DCB inhibition of cellulose synthesis is not clear. DCB is thought to act primarily on the stages of cellulose synthesis involving polymerization of Glc into a β-4-linked glucan. In an attempt to delineate the mechanism of action of DCB in cotton fibers, Delmer et al. (1987) used the photoreactive analog DCPA to identify an 18-kD DCB-binding protein thought to function as a regulatory protein for β-glucan synthesis in plants. This acidic protein was found primarily in the 100,000*g* supernatant and has not been further purified or characterized (Delmer and Amor, 1995). Labeling of UDP-Glc in vivo does not appear to be affected by DCB, indicating that its effect is at some later step in the cellulose synthesis process (Delmer and Amor, 1995). The second stage in cellulose microfibril formation involves β-4-linked glucan crystallization into cellulosic microfibrils at the PM. Mizuta and Brown (1992) have summarized the effects of DCB on the PM cellulose-synthesizing complex (the terminal complex) from higher plants and algae. These effects include a decrease or increase in the number of rosette-terminal complexes in *Funaria* and *Triticum*, respectively, and an inhibition of microfibril synthesis and assembly of *Vaucheria* terminal complexes.

It has recently been demonstrated that DCB inhibits synthesis of ECM polymers in the noncellulosic red mi-

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* Corresponding author; e-mail mrgretz@mtu.edu; fax 1–906–487–3167.

Abbreviations: ADCB, 3-amino-2,6-dichlorobenzonitrile; CFB, 2-chloro-6-fluorobenzonitrile; DC, 5-dimethylaminonaphthalene-1-sulfonyl chloride; DCB, 2,6-dichlorobenzonitrile; DCBF, 5-dimethylaminonaphthalene-1-sulfonyl-(3-cyano-2,4-dichloro)aniline; DCPA, 2,6-dichlorophenylazide; ECM, extracellular matrix; F/2, F/2 enriched seawater culture medium; NDCB, 3-nitro-2,6-dichlorobenzonitrile; PE, membrane pellet resulting from 100,000*g* centrifugation; PM, plasma membrane; Su_1, supernatant from 5,000*g* centrifugation of nondetergent buffer extract; Su_2, supernatant from 100,000*g* centrifugation of nondetergent buffer extract.

croalgae *Rhodella* (Arad et al., 1993) and *Porphyridium* (Arad et al., 1994). These investigators isolated DCB-resistant mutants with a significantly modified cell wall composition. We have discovered another example of DCB inhibition of ECM synthesis in the diatom *Achnanthes longipes* Ag., which has been characterized as noncellulosic by Wustman et al. (1997). We are currently investigating ECM assembly in this organism, because this pennate diatom is a prominent member of fouling communities in marine environments (Johnson et al., 1995), and we are interested in the mechanisms and compounds it utilizes for adhesion. Biofoulers can cause a variety of problems, including biocorrosion of submerged platforms, ship hulls, and other surfaces, and increased drag on ships, which results in fuel inefficiency (Alberte et al., 1992).

The ECM component most often associated with diatoms is the frustule, which is composed primarily of hydrated amorphous silica and consists of two overlapping halves (valves) and intervening structures called girdle bands. Although diatoms may attach directly via their frustules, other extracellular components are primarily responsible for adhesion. Most diatoms attach to surfaces via extracellular polymers extruded through a longitudinal slit in the valve (the raphe) or from an apical pore field (Hoagland et al., 1993). *A. longipes* is heterovalvar, with one raphid and one rapheless valve. Biosynthesis of extracellular polymers is an important part of an adhesion process that encompasses initial attachment, motility, and production of permanent adhesion structures (Gordon and Drum, 1970; Edgar and Pickett-Heaps, 1984; Hoagland et al., 1993). Motility within a biofilm provides a number of obvious and important ecological advantages, and several hypotheses concerning the mechanism of diatom motility involve the synthesis of extracellular polymers (Gordon and Drum, 1970; Edgar and Pickett-Heaps, 1984). Stalks and other extracellular structures that elevate diatoms above the substratum confer advantages in competition for nutrients and light within a biofilm (Johnson et al., 1995). Stalks of *A. longipes* usually consist of three regions: a surface-adhered pad, a collar associated with the frustule at a terminal nodule or apical pore field, and an intervening shaft that separates the cell from the substratum (Daniel et al., 1987).

Metabolic inhibitors such as DCB have proven useful for delineation of metabolic pathways and investigations of extracellular polysaccharide biogenesis in algae and higher plants (Delmer and Amor, 1995). In this study, we describe the reversible inhibition of the adhesion process in *A. longipes* by DCB and related compounds. An 18-kD polypeptide isolated from a membrane fraction of *A. longipes* labels with DCBF, an analog of DCB, which to our knowledge represents the first direct fluorescent probe for localization of DCB action.

MATERIALS AND METHODS

Chemicals

DCB and CFB were purchased from Aldrich, NDCB was from Maybridge Chemical Co. Ltd. (Cornwall, UK), and DC was from Molecular Probes (Eugene, OR). Fluorescent molecular mass standards were acquired from Sigma.

Plant Material

Achnanthes longipes Ag. was procured as described by Wustman et al. (1997) and cells were prepared for inoculation by blending for 10 s at low speed in seawater (10×), followed by washing with sterile seawater for 1 min, and centrifugation at 250*g* for 1 min (12×). Diatoms were further treated overnight in an antibiotic solution composed of 30 μg/mL kanamycin, erythromycin, and penicillin-G in F/2 (Guillard, 1975), followed by washing with sterile seawater several times before inoculation into flasks. Removal of all traces of antibiotic was required to achieve normal development. Diatoms were cultured in 100 to 200 mL of F/2 medium in 250- or 500-mL flasks maintained at 18°C on a 12-h:12-h light:dark cycle under 100 μmol m^{-2} s^{-1} cool-white fluorescent illumination. Cells were harvested after two weeks using aseptic techniques by scraping attached cells from the flasks with a plastic spatula, followed by washing with sterile seawater.

Electron Microscopy of Adhesives

Diatoms were allowed to adhere to small pieces of polymerized Spurr's resin immersed in F/2. Cells and associated adhesives were fixed sequentially in 1% (v/v) glutaraldehyde in filtered seawater for 12 h at 4°C, and then 1% OsO_4 (v/v) in seawater for 1.5 h at 23°C, followed by a graded series (10%) from seawater to distilled water. Prior to dehydration, some samples were treated with freshly prepared 5% (v/v) HF for 10 min at 23°C to dissolve the silicon frustules. Dehydration was carried out at 23°C by a graded series (20% steps) from distilled water to 100% ethanol, with 15 min of agitation at each step. Following three washes with 100% ethanol, diatoms were infiltrated with Spurr's resin by a graded series (25% steps) of washes from ethanol to 100% Spurr's resin, with 1 h of agitation at each step, and embedded following three changes with 100% Spurr's resin. Ultrathin sections were poststained for 10 min with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate and examined using a transmission electron microscope (model 301, Philips, Eindhoven, The Netherlands).

Monitoring the Adhesion Sequence

Two substrata were utilized to observe settlement and initial adhesion of *A. longipes*: (a) acid-washed (30% [v/v] HCl) glass (hydrophilic surface, air-bubble contact angle of <29° measured in filtered seawater as described by Fletcher and Marshall [1982]); and (b) polystyrene (hydrophobic surface, air-bubble contact angle >67°). Diatom attachment and stalk production on strips of substrata immersed in F/2 in glass Petri plates were monitored with an inverted microscope. Details of the adhesion sequence were obtained by transferring attached diatoms to a specially constructed observation chamber designed to accommodate glass strips immersed in F/2 and oriented parallel to the optical axis. The chamber was maintained at 18°C and approximately 100 μmol m^{-2} s^{-1} illumination with a homemade peltier-cooled stage fitted to a light microscope (Axioskop, Zeiss). Diatoms were viewed with a water-immersion lens (40X Achroplan, Zeiss). A video camera (DXC-930 3-CCD, Sony, Tokyo, Japan) provided output to a time-lapse video recorder (AG-6730, Panasonic, Secaucus,

NJ) and/or an active imaging digitizer board (SNP-24, Active Imaging, Berkshire, UK) in a work station (SUN SPARC 10, Sun Microsystems, Mountain View, CA).

Effect of DCB and Analogs on the Adhesion Sequence

DCB, CFB, ADCB, DCBF, or DCPA was dissolved in DMSO according to the method of Arad et al. (1994) and added to F/2 culture medium to yield 10, 50, 100, 150, or 200 μM, respectively, DCB or an analog and 0.3% (v/v) DMSO in F/2. Controls consisted of F/2, 0.3% (v/v) DMSO in F/2, or 400 μM DC in F/2. *A. longipes* was harvested at the beginning of the light period, mixed, and washed twice with sterile F/2 to produce single cells before inoculation into treatment chambers to a final concentration of approximately 2×10^4 cells/mL, and maintained under continuous illumination. DCB or an analog was added, and motility was assessed by microscopic observation of random fields of view as a percentage of moving versus nonmoving cells exhibiting raphe valve-substratum interaction. Stalk elongation was measured using a camera lucida with a digitizing tablet (JS-2, Jandel, Carle Madera, CA) and software (Sigma Scan, Jandel). Each value represented a mean of three replicas, with each replica consisting of counts of at least 50 attached cells. To maintain active extracellular adhesive synthesis, and because of an observed tendency of DCB and analogs to precipitate slightly from marine growth medium, long-term exposures to DCB and analogs were conducted by periodically replacing DCB-containing medium with "preconditioned" F/2 (filter-sterilized culture medium in which *A. longipes* had been previously cultured at high cell densities for at least 6 h) with DCB freshly added. To measure reversibility, chambers were flushed with sterile F/2 five times and medium was replaced with preconditioned F/2 lacking inhibitors. Cell growth was assessed by cell counts of random fields of view at various times after inoculation.

Effect of DCB on Extracellular Polysaccharide Synthesis

Cells from 1-week-old cultures that had been optimized for production of extracellular polymers were harvested, divided into three equal portions, and washed with sterile seawater. Portion I was mixed into preconditioned F/2 medium and immediately prepared for analysis. Portion II was inoculated into preconditioned F/2 medium and cultured under the conditions described above for 48 h. Portion III was incubated in preconditioned F/2 medium containing 150 μM DCB for 48 h. Preparation of the portion for analysis was accomplished by separating the culture medium from cells with associated extracellular materials by centrifugation (250*g* for 5 min). The culture medium was concentrated by flash-evaporation followed by exhaustive dialysis against distilled water and freeze-drying. Extracellular materials and cells were chemically fractionated as described by Wustman et al. (1997). Hot water-soluble, hot water-insoluble/hot $NaHCO_3$-soluble, and hot water-insoluble/hot $NaHCO_3$-insoluble fractions were dialyzed against distilled water and freeze-dried. Total sugar content was estimated by the phenol sulfuric acid reagent (Dubois et al., 1956) with Glc as standard; protein was estimated with a protein assay reagent (Bio-Rad) using BSA as standard. Monosaccharide composition was determined according to the method of Wustman et al. (1997).

Preparation of ADCB, DCBF, and DCPA

NDCB was reduced to ADCB as follows: NDCB (1 g) was added gradually to 9 mL of 12 N HCl containing 2 g of $SnCl_2 \cdot 2H_2O$ at 23°C; the mixture was maintained at 70°C for 20 h and 100°C for 4 h and cooled to 1°C; whereupon 50 mL of $CHCl_3$ was added. The organic phase was discarded and 25 mL of 1 N NaOH and then 100 mL of $CHCl_3$ was added to the aqueous phase, mixing after each addition. ADCB partitioned into the organic phase, which was applied to a silica gel column (70–230 mesh, 60Å, 2.5 × 60 cm) and eluted with $CHCl_3$. Fractions from the column were monitored with silica gel TLC (mobile phase $CHCl_3$). ADCB was a white solid that was soluble in HCl. Electron-impact MS yielded diagnostic ions at 186 (molecular ions), 151, and 124. ^{1}H-NMR yielded a shift at approximately 4.4 parts per million, which corresponds to the amino group. DC was coupled to ADCB using methods modified from Wiechmann (1977). ADCB (1 mg) dissolved in 1 mL of $CHCl_3$ was added to 1 mL of $CHCl_3$ containing 10 mg of DC; 250 μL of 0.1 M $NaHCO_3$ was added and stirred at 5°C for 20 h. The organic phase was applied to a silica gel column (70–230 mesh, 60Å, 2.5 × 100 cm) and DCBF was eluted with $CHCl_3$. Fractions from the column were monitored with silica gel TLC (mobile phase $CHCl_3$). Purified DCBF yielded a distinct fluorescent spot when exposed to UV. DCPA was synthesized following the methods of Cooper et al. (1987).

Protein Preparation and Labeling

Diatom cells actively synthesizing extracellular adhesives were harvested, washed twice with filtered seawater, and centrifuged at 1,000*g* for 10 min. The pellet was frozen with liquid N_2 and ground to a powder with a chilled mortar and pestle. The powder was extracted by two methods: (a) SDS buffer (100 mM Tris-HCl [pH 7.5] containing 1 mM EDTA-Na_2, 2% [w/v] SDS, and 100 mM DTT) was added to the powder and the insoluble materials were sedimented by centrifugation at 5,000*g* for 10 min. The supernatant was concentrated to 1 mg/mL protein by ultrafiltration, and 20 μL of this solution was added to an equal volume of 125 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 20% (v/v) glycerol, and 100 mM DTT; or (b) nondetergent buffer consisting of 100 mM Tris-HCl (pH 7.0) containing 100 mM DTT, 1% (w/v) PVP, and 5 mM PMSF was added to the powder and insoluble materials were sedimented by centrifugation at 5,000*g* for 10 min. Su_1 was centrifuged (100,000*g*) at 4°C for 1 h to sediment membranes, yielding Su_2 and PE. Su_1 and Su_2 were each added to an equal volume of 125 mM Tris-HCl (pH 6.8) containing 4% (w/v) SDS, 20% (v/v) glycerol, and 100 mM DTT. The PE was suspended in 125 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol, and 100 mM DTT. After boiling for 4 min and centrifugation (10,000*g*, 10 min), 10 μL of each fraction was loaded in a lane. The buffer system of Laemmli (1970) was used with a 15% acrylamide separation gel and a 6% stacking gel in a vertical mini-slab gel apparatus with

Figure 1. Time-lapse microscopy of the adhesion sequence. Valve (top) view (A) and girdle (side) view (B) of a motile cell showing the two overlapping valves (likened to the top and bottom of a Petri plate) of the heavily silicified cell wall or frustule. Cells move irregularly forward, backward, or in a curving or rotatory manner. Jerky, sudden acceleration and deceleration occur alternately within the first 6 h after inoculation into new media. Motile cells always maintain their raphid valve in close contact with the substratum. Eventually, cell motility ceases and a pad is formed from one polar region of the raphid valve face (C) (the central ribbon appears as a line in the center of shaft [arrow in I]). Some of the pad material remains attached to the cell forming a collar. The shaft elongates (D–I), which separates the cell from the substratum. Usually shaft elongation occurs at a rate of approximately 7 μm/h, but occasionally there is a disruption in deposition, causing a discrete thickening in the stalk (arrowhead in G; interruption occurred in this sequence at E). Attached cells undergo successive cell divisions to form chain-like multiple cell stacks attached to the substratum proximally via the stalk (J–FF). (Legend continues on facing page.)

0.75-mm spacers (Hoefer Scientific Instruments, San Francisco, CA). Following electrophoresis at 25 mA for 2 h, gels were stained with 1.25% (w/v) Coomassie blue R-250 in 50% (v/v) methanol:10% (v/v) acetic acid for 20 min. Destaining was accomplished with 50% (v/v) methanol: 10% (v/v) acetic acid for 2 h.

Unstained gels were labeled with 200 μM DCBF dissolved in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 2% (w/v) SDS, and 0.3% (v/v) DMSO for 30 min in the dark. Labeled gels were washed with several changes of 50 mM Tris-HCl (pH 7.5) (approximately 2 h) and observed using UV illumination. Controls included gels pretreated with 400 μM DCB in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 2% (w/v) SDS, and 0.6% (v/v) DMSO prior to DCBF labeling, and gels labeled with 400 μM DC in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 2% (w/v) SDS, and 0.3% (v/v) DMSO in the dark.

RESULTS

Exopolymer-Mediated Adhesion Sequence

Initial adhesion of *A. longipes* inoculated into sterile F/2 is in part dependent on the substratum. Hydrophobic surfaces promote nonspecific passive (reversible) initial attachment characterized by a loose association with random orientation of the diatom in relation to the substratum. Frustules lacking protoplasm and cells pretreated with DCB or general metabolic inhibitors also exhibit this type of "passive" adhesion, indicating that active synthesis of extracellular polymers is not required. In contrast, diatoms inoculated onto hydrophilic substrata do not attach unless they are actively secreting exopolymers. These exopolymers participate in "active" adhesion, as shown by raphe-mediated motility and subsequent formation of permanent attachment structures such as stalks. Active adhesion can also be observed on hydrophobic surfaces following sufficient time of association of diatoms with these surfaces.

The active adhesion process (briefly described by Daniel et al., 1987) can be broken down into four phases, as observed in Figure 1. Below is a brief overview of these events (for details, see Wang, 1995).

Phase I

Initial active adhesion of *A. longipes* on hydrophilic surfaces is achieved by raphe-associated transient attachment. Soon after cells contact the substratum, they reorient themselves so that the raphid valve is proximal to the substratum (Fig. 1, A and B). The cells move smoothly for a short period, stop, and then reverse direction. Sudden changes in velocity, careening movements about the transapical plane, and trembling movements are also seen. Movement is usually in a straight line, but occasionally it is curved or rotatory. When the substratum is not flat, motile cells can change directions to move around or over obstacles or bridge gaps. Upon inoculation into sterile F/2, cells distance themselves from one another before producing more permanent attachment structures, leading to a lawn of stalked diatoms.

Phase II

Cell motility eventually ceases (approximately 6 h after inoculation into sterile F/2 medium) and small globular structures (pads) are produced, attaching the cell to the substratum (Figs. 1C and 2A). These pads are amorphous and may serve as a transitional state between the secretion of polymers related to motility and the formation of the shaft.

Phase III

Following basal pad formation, a highly structured shaft is secreted from the same pole of the raphid valve (Fig. 1, C–I). During this process, active vesicle movements are observed at cell apices. Shafts are elongated, flexible, multilayered structures connecting cells to pads (Fig. 2, B–D). Multiple fibrillar layers oriented both longitudinally and transversely to the long axis of the shaft are evident when shafts are fixed and dehydrated for electron microscopy (Figs. 2 and 3). The shaft elongation rate is approximately 7 μm/h based on time-lapse video microscopy. During development of the shaft, a collar is also formed near the valve (Fig. 1O). Most attached cells produce stalks 100 to 200 μm in length within 48 h of inoculation. Interruptions in shaft synthesis result in thickenings in the shaft and/or a ring of collar-like material (Fig. 1G, arrowhead). Occasionally, cells produce two stalks, one from each polar region of the raphid valve.

Phase IV

In preparation for cell division, cell expansion occurs with concomitant synthesis of girdle bands (Fig. 1, J–K and R–Z) between the two valves. Because of the rigid ECM, the plane of cell division is perpendicular to the colony long axis, and successive cell divisions result in a filamentous colony or a stack of cells (Fig. 1, J–EE) averaging 1 to 2 mm in length. This is similar to the process in other raphid diatoms such as *Nitzschia* (Round et al., 1990). Under optimal conditions, cells throughout stacks divide almost synchronously, resulting in a pattern of cell junctions alternating between two immature valves and two mature valves throughout the colony (Fig. 1BB). Cells acquire the ability to generate raphe-associated motility only at mature valve interfaces, resulting in two (or more) cell units (Fig. 1FF) leaving the colony. These paired cells then separate into single cells (Fig. 1HH) to begin the sequence anew. Extracellular adhesive synthesis and cell division appear to be

(Legend continued from facing page.) Formation of a cleavage furrow cutting the cell in two (K–M, U–W, Y–AA) occurs within 3 to 4 min. During each division, only one new or "immature" valve is constructed for each new cell. The formation of new valves is completed within 20 min (L–Q). Cells divide almost synchronously (V–EE), forming long stacks with alternating "immature" and "mature" valve junctions. Cells eventually gain the ability to dissociate themselves from the stack. This always occurs at the junction of two mature valves (FF). The resulting two or multiple cell units may be motile for a time until they, too, separate into single cells (HH) to begin the sequence again. cl, Collar; iv, immature valve; mv, mature valve; p, pad; s, shaft. Scale bar = 10 μm.

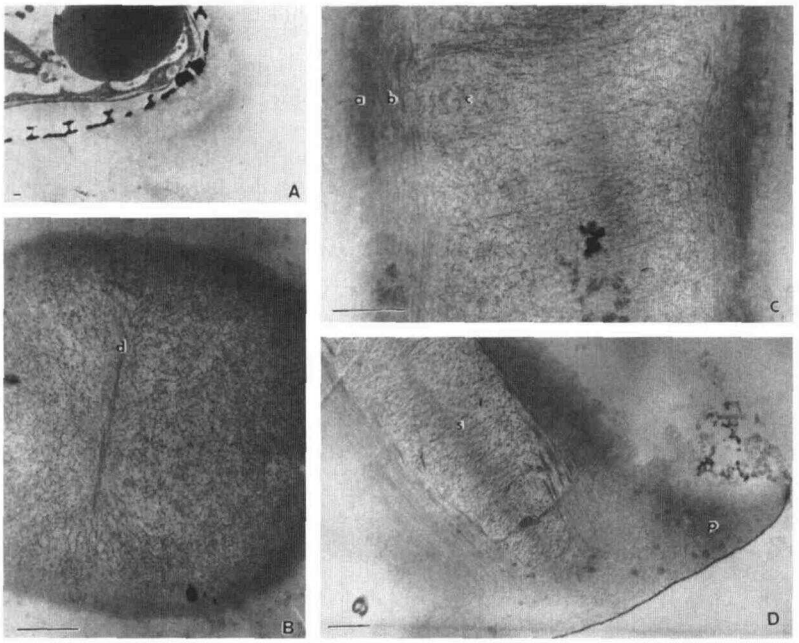

Figure 2. Electron microscopy of extracellular adhesives. A, Polar region of cell with associated pad. B, Transverse section through the shaft showing core ribbon of fibrillar material (d). Upon extrusion, this ribbon is oriented parallel to the raphe. C, Longitudinal section of the shaft showing layers a through c, as defined in Figure 3. D, Stalk-substratum interface and basal region of shaft (p, pad; s, shaft). Scale bars = 1 μm.

mutually exclusive events in *A. longipes*, since we did not observe cell division coincident with extracellular adhesive synthesis. The average rate of cell division was once in each 12-h:12-h light:dark cycle, and no direct correlation between day-night cycle and time of cell division was observed. Cell density and/or some factor (possibly N limitation) inherent in preconditioned culture medium influenced the frequency of cell division, with fresh culture medium and low cell densities favoring cell division over extracellular adhesive production.

Ultrastructure of Adhesives

EM of stalks (Fig. 2) revealed a complex, circular, multilayer organization throughout the shaft. As summarized in Figure 3, the thickness of the four layers, the orientation of the fibrils, and the distribution of amorphous materials are clearly distinguished. In sharp contrast, the pad is composed of an amorphous material that appeared to be continuous with the external layer of the shaft (Fig. 2D).

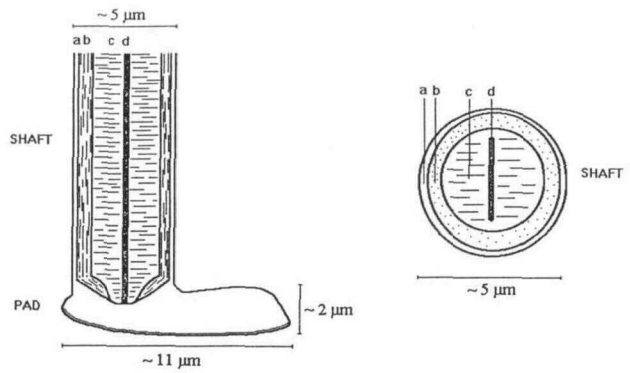

Figure 3. Schematic representation of the stalk based on light and electron microscopic observations. Left, Longitudinal cross-section; right, transverse cross-section. The stalk is composed of two major zones, the shaft and the pad. Within the shaft several layers can be distinguished: layer a is external, loosely associated, relatively unstructured; layer b, after fixation and dehydration for electron microscopy, appears fibrillar and multilayered with fibrils parallel to the long axis of the shaft; layer c appears fibrillar with perpendicular orientation relative to long axis; and layer d is the core ribbon with densely packed fibrils perpendicular to the long axis of the shaft.

Inhibition of Extracellular Adhesive Synthesis by DCB and Analogs

A DCB concentration range between 10 and 200 μM was tested. Extracellular adhesive synthesis was sensitive to

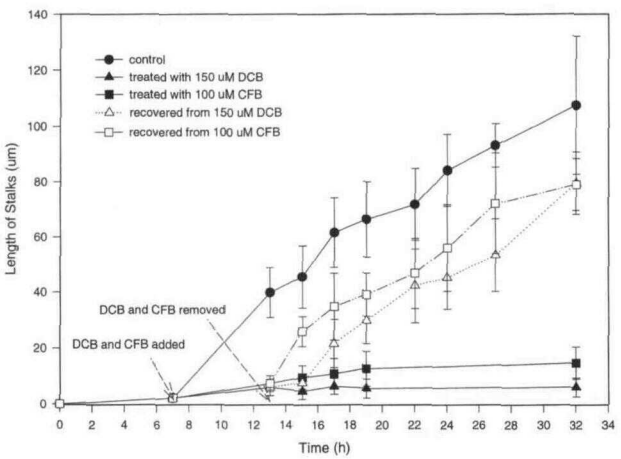

Figure 4. DCB and CFB interrupt the sequence of adhesion by inhibiting synthesis of extracellular adhesives. Approximately 7 h following inoculation into sterile culture medium, *A. longipes* cells begin to synthesize stalks. Addition of DCB or CFB at 7 h prevents stalk elongation. The effect is reversible; upon removal of inhibitor at 13 h and introduction of preconditioned culture medium, stalk elongation resumes at rates comparable to controls. DMSO (0.3%, v/v) in culture medium gives the same result as control (F/2 culture medium; data omitted for clarity). Values represent three replicas, each replica consisting of counts of at least 50 cells. Error bars = SD.

10 μM DCB and the most significant effects occurred at 100 to 150 μM. A positive correlation between DCB concentration in the range of 10 to 150 μM and the extent of inhibition of stalk production and motility was noted. Decreased motility was observed immediately upon inhibitor introduction. When maintained in 150 μM DCB for long periods by exchange of medium, cells did not produce adhesives but continued to divide, and the resultant 2-week-old culture consisted of very long stacks of cells unattached to the substratum. In these cultures, colony morphology and frustule morphology was not obviously distinct (at the light microscope level) from that of untreated controls.

When *A. longipes* cells were inoculated into sterile culture medium, they remained motile for approximately 6 h and then began to produce stalks (Fig. 4). If treated with 150 μM DCB during this motile phase, approximately 50% of the cells became nonmotile within 1 h, and the remainder exhibited a substantial decrease in velocity. If DCB treatment was extended to 24 h all cells became nonmotile. As long as DCB treatment continued, stalk formation was not initiated. Inhibition of extracellular adhesive synthesis in the presence of DCB and CFB was reversed upon removal of inhibitors. When DCB or CFB was added at 7 h following inoculation, stalk synthesis was delayed until the inhibitor was removed (Fig. 4). When culture medium containing DCB or CFB was replaced with preconditioned inhibitor-free medium, stalk production resumed with only a short delay and rates of stalk production quickly reached that of control cells (Fig. 4). When culture medium containing DCB was replaced with sterile F/2 medium, reinitiation of stalk production was delayed up to 6 h and then progressed at rates comparable to controls. This effect may be due to the well-documented effect of nitrogen limitation on polysaccharide synthesis in diatoms (Myklestad and Haug, 1972).

In the same manner as for DCB, 100 to 150 μM ADCB, CFB, or DCBF were most effective at inhibiting motility and stalk synthesis; reduced concentrations produced more limited effects (data not shown). Diatoms treated with >200 μM concentrations of any of the above inhibitors became obviously unhealthy within 1 d and ceased to divide. DCPA proved to be an effective inhibitor of adhesive synthesis at 10 μM, and at 150 μM caused cell death within 1 d. *A. longipes* growth rates and extracellular adhesive synthesis were not affected by control treatments of 0.3% DMSO in F/2 or DC in F/2.

That 150 μM DCB effectively inhibited synthesis of all polysaccharides external to the frustule during 48 h of exposure was indicated by analysis of total sugar content and monosaccharide profiles. Culture medium and the hot water-soluble and hot $NaHCO_3$-soluble fractions from the DCB-treated (portion III) preparation showed no increase in total sugar content and no change in constituent monosaccharides compared with the control (portion I). In contrast, a dramatic increase in total sugar content was observed in the hot $NaHCO_3$-soluble fraction after 48 h of incubation of diatoms in preconditioned F/2 culture medium (portion II).

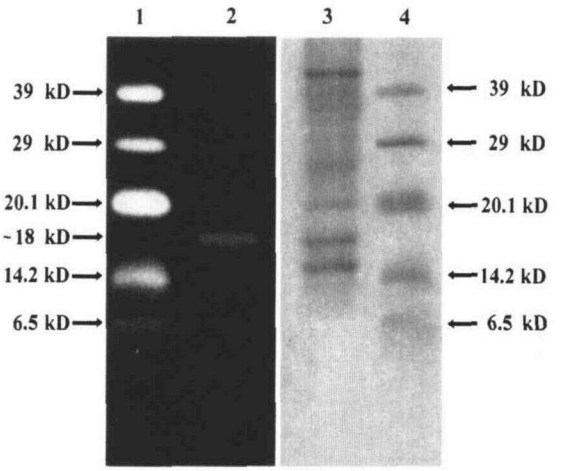

Figure 5. SDS-PAGE of cellular proteins from *A. longipes* labeled with fluorescent DCB analog (DCBF, lane 2) and a corresponding lane stained with Coomassie blue (lane 3). Lanes 1 and 2 were visualized with UV irradiation. Molecular mass markers (lanes 1 and 4) are: alcohol dehydrogenase, 39 kD; carbonic anhydrase, 29 kD; trypsin inhibitor, 20.1 kD; α-lactalbumin, 14.2 kD; aprotinin, 6.5 kD.

Identification of a DCB-Binding Polypeptide

When *A. longipes* cells actively synthesizing adhesives were frozen, powdered, and extracted with SDS, an 18-kD polypeptide that preferentially interacted with DCBF was identified using SDS-PAGE (Fig. 5). The DCBF labeling procedure was efficient and results were instantly available, since the probe could be readily visualized on a UV light box. DCBF migrated at the separating gel front if incubated with the protein extract before electrophoresis, indicating that association of DCBF with the polypeptide is not due to a covalent interaction. A substantial reduction in the intensity of labeling was noted when gels were incubated with 400 μM DCB prior to DCBF labeling; gels treated with 400 μM DC alone did not show a fluorescent band under UV irradiation. When we applied DCBF to gels containing polypeptides that had been isolated from mechanically purified stalks (not shown), no labeling was observed and an 18-kD polypeptide was not found.

When extractions were carried out without detergent and the extract fractionated, a crude membrane fraction PE was highly enriched in the 18-kD polypeptide, although total protein yield was low. After insoluble stalks and frustules were eliminated by centrifugation at 5,000*g* for 10 min, the Su_1 retained a small amount of 18-kD protein that labeled with DCBF. Upon subfractionation of Su_1 with centrifugation at 100,000*g* for 1 h to pellet membrane components, SDS-PAGE of SDS-solubilized PE revealed the 18-kD polypeptide, although it was not detected in Su_2. This suggests that the 18-kD DCB-binding polypeptide may be (or be a subunit of) a membrane-associated or integral membrane protein.

DISCUSSION

We have discovered that the herbicide DCB and its analogs CFB, DCPA, ADCB, and DCBF inhibit synthesis of

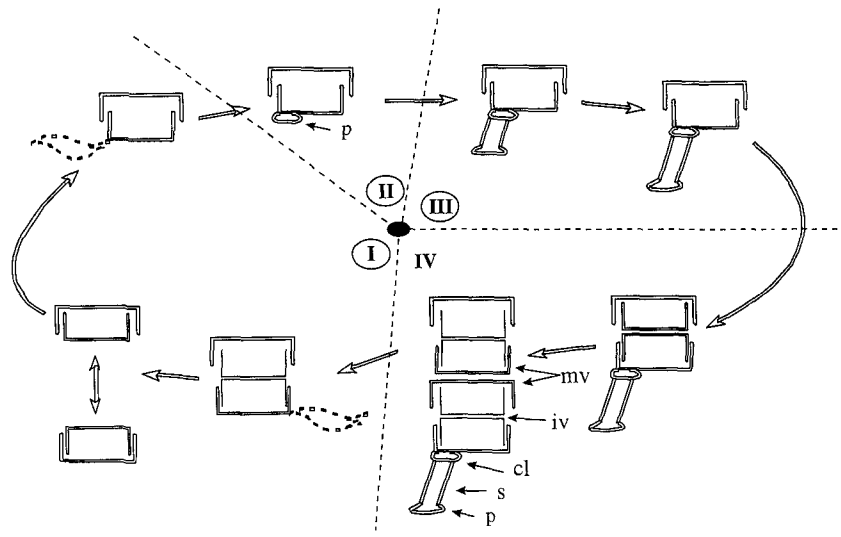

Figure 6. A diagram summarizing the adhesion sequence of *A. longipes* on hydrophilic surfaces. Adhesion occurs in four phases: I, raphid-valve-associated motility; II, formation of the first stalk component, the pad, from one pole of the raphid valve; III, synthesis of a shaft that elevates the diatom above the substratum; and IV, cell division. DCB and analogs inhibit the adhesion sequence in phases I through III (circled). cl, Collar; iv, immature valve; mv, mature valve; p, pad; s, shaft.

noncellulosic extracellular polysaccharides critical for motility and stalk production in the marine diatom *A. longipes* (summarized in Fig. 6). Wustman et al. (1997) reported no evidence of cellulose in the extracellular polymers from *A. longipes*, so ours represents only the second report to our knowledge of DCB inhibition of synthesis of noncellulosic polysaccharides, indicating that DCB is not a specific inhibitor of cellulose synthesis in marine algae. The first reports (Arad et al., 1993, 1994) found that DCB inhibited polysaccharide production in two noncellulosic red algal unicellular genera, *Rhodella* and *Porphyridium*, that protoplast regeneration was inhibited by DCB, and that the ECM polysaccharides of DCB-resistant mutants were modified in having higher proportions of methyl galactosyl residues (*Rhodella*) or xylosyl residues (*Porphyridium*).

The concentration of DCB required to disrupt ECM assembly in red algal unicells and extracellular polysaccharides in *A. longipes* was approximately 5 to 10 times that required for effective inhibition of cellulose synthesis in most higher plants. Studies of effects of DCB applied as an herbicide indicate that DCB can be metabolized to compounds that are uncouplers of oxidative phosphorylation over a period of several hours (see review by Montezinos and Delmer [1980]). The rate of stalk production (approximately 6–7 μm/h) makes it difficult to evaluate the short-time-period effects of DCB on this process; however, effects on motility and pad production are observable within minutes of exposure to 10 μM DCB. Also, our results with long-term exposure (2 weeks) of *A. longipes* to 150 μM DCB indicate no effect on cell division or frustule formation, arguing against inhibition of a general metabolic process as an explanation for DCB blockage of extracellular polysaccharide adhesive synthesis. An explanation for the higher concentration of DCB necessary for inhibition of extracellular polysaccharide synthesis in marine plants was advanced by Arad et al. (1993) as having to do with precipitation of the DCB from the marine growth medium, which would have the effect of increasing the apparent concentration necessary to generate a response. We have also observed a slight solubility problem with DCB and its analogs in seawater-based growth media (see "Materials and Methods"). Maximal inhibition of extracellular adhesive synthesis in *A. longipes* occurs at 10 μM DCPA; higher concentrations are toxic. DCPA was more soluble than DCB in our growth medium. We recently observed that 5 to 10 μM DCB is sufficient to inhibit stalk formation in the freshwater diatom *Cymbella cistula* (Q. Sun and M.R. Gretz, unpublished data), which, again, may relate to solubility. We observed no adverse effects from the DMSO utilized to solubilize DCB and its analogs, although low concentrations of DMSO have been reported to give significant inhibition of long-term growth in cell lines of tobacco and tomato (Shedletzky et al., 1992).

Our observations reveal that generation of the beautiful, architecturally complex silicious frustule (cell wall) of *A. longipes* does not appear to be affected (at light microscopic resolution) by DCB and its analogs at concentrations adequate to completely inhibit extracellular adhesive polysaccharide production. This is in contrast to its effects in a long list of other organisms, including red algal unicells (Arad et al., 1993, 1994), pea internodes (Edelmann and Fry, 1992), *Zinnia* tracheary elements (Taylor and Haigler, 1993), and cultured cells of tomato and tobacco (Shedletzky et al., 1992; Wells et al., 1994), in which DCB promotes significant alterations to the cell wall chemistry and structure. Although diatom frustules are composed primarily of hydrated amorphous silica, there is an organic portion termed the diatotepum that has a polysaccharide component containing uronosyl residues (see review by Hoagland et al. [1993]). Radiotracer studies indicate that in some diatoms this polysaccharide is added after silicon deposition is complete (Coombs and Volcani, 1968), and so possibly is not necessary for normal frustule formation. Several models have been advanced for the mechanism of amorphous silica deposition in characteristic intricate patterns during frustule formation (see review by Round et al. [1990]), including one that invokes a polysaccharide template within the lumen of the silicon deposition vesicle upon which silicification occurs (Pickett-Heaps et al., 1979). Synthesis of this polysaccharide template in *A. longipes*, if it exists, is not inhibited by DCB and its analogs, since frustules are not distinguishable from

those synthesized in the absence of inhibitors at the light microscopic level. The absence of a significant effect of DCB and its analogs on the process of cell wall (frustule) formation in *A. longipes* would indicate that the mechanisms of biogenesis are, at least in part, unique from those involved in the synthesis of extracellular adhesives involved in motility and stalk formation. We are currently performing detailed analyses of cell wall formation in the presence of DCB and its analogs in the hope that it may yield interesting clues to the importance of polysaccharide participation in the fabrication of the intricate frustule of *A. longipes*.

Nuclear division, cytokinesis, and concomitant frustule biogenesis appear to be unaffected by DCB for many generations (14+ cell divisions), although extracellular adhesive synthesis is totally inhibited. Unlike higher plants and most algae, which require polysaccharide synthesis (cellulose) for maintenance of cell shape and rigidity, the method we used for the study of the effects of DCB on extracellular adhesive polysaccharide synthesis appears to be independent of the effects on cell propagation and growth, since the polysaccharide synthesis inhibited by DCB in diatoms is not essential for the maintenance of normal cell morphology. *A. longipes* is a convenient organism for the study of DCB's action on polysaccharide synthesis because large numbers of cells can be rapidly cultured for metabolic studies or to provide large quantities of DCB-receptor polypeptides for further study.

DCBF, a fluorescent analog of DCB, represents a powerful new probe for investigating the mode of action of DCB and related compounds on polysaccharide synthesis. DCBF is easily synthesized in a two-step procedure from NDCB and DC, and can easily be separated from contaminants by silica gel chromatography. Although DCBF inhibits polysaccharide synthesis with the same effectiveness as DCB, DC shows no effect on growth, motility, or stalk production by *A. longipes*, implying that only the DCB part of the molecule confers activity. Previous studies have established that herbicide activity is retained if DCB analogs retain a planar structure and contain halogen groups at positions 2 and 6 of the benzene ring (refs. cited by Delmer et al., 1987). The analogs CFB, ADCB, DCPA, and DCBF meet these criteria. ADCB and DCBF also carry substitutions at position 3 of the benzene ring. Since these compounds were as effective on a molar basis as DCB at inhibiting extracellular polysaccharide assembly in *A. longipes*, we conclude that substitution at C-3 does not significantly interfere with activity.

Applying DCBF directly to SDS gels, we detected an 18-kD polypeptide in diatoms actively synthesizing extracellular adhesive polysaccharides, which may represent a receptor for this herbicide. This 18-kD polypeptide is found associated with the membrane fraction in vivo, and preliminary experiments with native gels (not shown) indicate that it may be a subunit of a larger protein in its native state. The relationship of this 18-kD polypeptide to the apparent DCB receptors described from cotton (18 kD), tomato (12 kD), and *Chara* (12 kD) (Delmer et al., 1987) is not clear, although inhibition of polysaccharide synthesis in these organisms (Pillonel and Meier, 1985; Foissner, 1992; Shedletzky et al., 1992; Wells et al., 1994) in response to DCB parallels our results with *A. longipes*.

Models of the mechanism of raphe-associated motility in diatoms consistently invoke polymer (mucilage) synthesis across the PM (Edgar and Pickett-Heaps, 1984; Gordon, 1987; Pickett-Heaps et al., 1991; Cohn and Disparti, 1994). The motive force has been postulated to be generated by the capillary force of the raphe fluid or by interactions of secreted mucilage strands with cytoskeletal components with suitable membrane mediation. Polysaccharide extrusion from diatoms in the form of stalks and chitin fibrils is also thought to be from PM-associated structures (reviewed by Hoagland et al. [1993]). In *A. longipes*, both stalk production and motility are inhibited by DCB and its analogs, which supports the concept that both processes are dependent on polysaccharide synthesis, and therefore may be related metabolically. Reports that labeling of UDP-Glc is not affected by DCB (Delmer and Amor, 1995) and evidence of DCB perturbation of PM intramembranous particle distributions (summarized by Mizuta and Brown [1992]), coupled with our finding of a membrane-associated DCBF receptor, have prompted us to speculate that DCB may exert its effect on the later steps in the extracellular adhesive synthesis pathway of *A. longipes*, perhaps at the PM. We are currently focusing on using DCBF as an intracellular probe to localize the membrane with which the DCBF-binding polypeptide is associated, with the intent of clarifying the interaction between PM, polysaccharide synthesis, and the extrusion of adhesive polymers.

The finding that the site of DCB inhibition may be intracellular is supported by results of a preliminary investigation of *A. longipes* adhesion to surfaces containing DCB. We have obtained a DCB-functionalized polyimide film (a polyimide backbone with short amide linkers to DCB) and coated this film on glass slides. When cultured in plates containing this film and glass substrata, diatoms colonize both surfaces and appear to be able to perform all phases of the adhesion process, including motility and stalk production on the DCB-doped film. This would indicate that DCB immobilized in the substratum is not effective at inhibition of adhesive production, and provides support for an intracellular location of DCB action in the process of adhesive production.

Active synthesis of extracellular polymers is not required for the initial nonspecific transient association of *A. longipes* with hydrophobic surfaces. The overall process of passive and/or active adhesion has similarities to the initial nonspecific reversible interaction of bacteria with surfaces that leads to an irreversible binding and specific attachment with production of extracellular polymers (van Loosdrecht et al., 1990). Although the importance of extracellular polymers in diatom attachment phenomena is well documented (Hoagland et al., 1993), the disruption of extracellular polysaccharide synthesis in *A. longipes* and the resulting inability to attach to substrata and change in colony morphology emphasizes the critical role these polymers play in the development of morphologies observed in nature. Lectin labeling and staining studies indicate a similarity in composition between pad material formed at the termination of motility

(just prior to shaft production) and extracellular polymers formed between the valves just prior to separation at the initiation of motility (Wustman et al., 1997). Our finding of inhibition of motility (and separation of the colony into individual cells) by DCB indicates that polysaccharide synthesis is required for motility and shaft production. Production of extracellular polysaccharide adhesives appears to be a crucial part of the developmental ecology of *A. longipes* in that it is critical for dispersion of cells from filamentous colonies as well as for adhesion to substrata. Although we routinely observed sexual reproduction in our cultures, we have not yet monitored the effects of DCB on this process.

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